Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition

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Summary

Mammalian spermatozoa undergo a series of molecular and biochemical changes collectively termed capacitation prior to acquiring the ability to fertilise the oocyte. Although phosphorylation of sperm proteins on tyrosine residues has been recognised as an important component of this process, the precise relationship between the phosphorylation status of mammalian spermatozoa and their capacity for fertilisation has remained unclear. In this study we demonstrate a causal relationship between tyrosine phosphorylation in spermatozoa and sperm-zona interaction. The phosphotyrosine expression associated with sperm capacitation localised to internal flagellar structures in permeabilised cells but could also be detected on the exterior surface of the sperm head in live cells. Importantly, almost all spermatozoa bound to the zona pellucida demonstrated this pattern of phosphoprotein localisation, compared to fewer than 15% of the free-swimming population. These data suggest that tyrosine phosphorylation plays a significant role in remodelling the sperm surface, so that these cells are able to recognise the zona pellucida. Phosphoproteome analysis yielded the first evidence of molecular chaperones, endoplasmin (erp99) and heat shock protein 60 (hsp60), as targets for phosphorylation on the surface of mouse spermatozoa, whereas immunofluorescence localised these proteins to the precise region of the sperm head that participates in zona recognition. Based on these results, we propose a novel mechanism for mammalian gamete interaction whereby the activation of sperm-surface chaperones by tyrosine phosphorylation during capacitation may trigger conformational changes facilitating the formation of a functional zona pellucida receptor complex on the surface of mammalian spermatozoa.

Key words: Fertilisation, Signal Transduction, Sperm, Capacitation, Phosphorylation, Chaperone

Introduction

Over 50 years ago independent reports suggested that ejaculated spermatozoa require a period of residence in the female reproductive tract before being capable of fertilisation (Austin, 1952; Chang, 1951). The changes that take place during this time confer upon spermatozoa the ability to adhere to the zona pellucida, undergo the acrosome reaction and initiate oocyte fusion, and have been collectively termed ‘capacitation’. Capacitation encompasses changes in intracellular pH, alterations in membrane lipid architecture and protein distribution, and initiation of complex signal transduction pathways (Baldi et al., 2002; Visconti and Kopf, 1998; Visconti et al., 2002). The observation that capacitation can be achieved in vitro has facilitated detailed investigation of these events.

Spermatozoa are transcriptionally inactive and thus rely heavily on post-translational modifications such as phosphorylation to regulate their cellular processes, including capacitation. Capacitation-dependent decreases in sperm phosphodiesterase activity (Monks and Fraser, 1987; Stein and Fraser, 1984) in concert with increases in adenylyl cyclase activity (Leclerc and Kopf, 1995; Morton and Albagli, 1973), intracellular cyclic adenosine monophosphate (cAMP) concentration (Hyne and Garbers, 1979; White and Aitken, 1989) and protein kinase A (PKA) activity (Visconti et al., 1997), suggest that a cAMP-PKA-dependent signalling cascade is active in the mature gamete. Uniquely in the male germ line, this signal transduction cascade results in the downstream induction of tyrosine kinases and phosphorylation. Phosphotyrosine expression during capacitation has now been demonstrated in the spermatozoa of numerous mammalian species (Aitken et al., 1995; Galantino-Homer et al., 1997; Kalab et al., 1998; Lewis and Aitken, 2001; Mahony and Gwathmey, 1999; Pommer et al., 2003; Pukazhenthi et al., 1996; Visconti et al., 1995a). A number of key regulators in this process have been identified, including calcium, bicarbonate, cholesterol and reactive oxygen species (Aitken et al., 1995; Aitken et al., 1998; Cross, 1998; Luconi et al., 1996; Visconti et al., 1995a; Visconti et al., 1999). Although tyrosine phosphorylation has been recognised as an important factor in capacitation, the precise relationship between the phosphorylation status of mammalian spermatozoa and their capacity for fertilisation has not yet been elucidated.

A capacitation-dependent increase in the tyrosine phosphorylation of proteins localised to the sperm flagellum has been demonstrated in a number of species (Ecroyd et al., 2003b; Mandal et al., 1999; Turner et al., 1999; Urner et al., 2001). This phosphorylation has been implicated in the onset of hyperactivated motility, one of the hallmarks of sperm capacitation (Mahony and Gwathmey, 1999; Nassar et al.,

1999; Si and Okuno, 1999). However, phosphorylation of proteins on the sperm tail cannot explain another key feature of capacitation, the ability of spermatozoa to interact with the zona pellucida and undergo acrosomal exocytosis. The significance of tyrosine phosphorylation in regulating the ability of capacitated spermatozoa to interact with the egg is therefore still unresolved.

In light of this uncertainty, we examined the relationship between protein tyrosine phosphorylation and the ability of mouse spermatozoa to target the zona pellucida. The localisation of phosphorylated proteins on fixed and live mouse spermatozoa during capacitation and zona pellucida interaction has been examined, and the identity of key phosphoproteins on the surface of spermatozoa established. As a consequence of these studies, we propose a novel hypothesis describing the mechanisms by which the tyrosine phosphorylation events associated with sperm capacitation regulate the ability of these cells to interact with the surface of the oocyte.

Materials and Methods

Reagents

HEPES, penicillin, and streptomycin were obtained from Gibco (Paisley, UK). Bovine serum albumin (BSA) and 3-[(3-Cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS) were from Research Organics (Cleveland, OH). Pregnant mare's serum gonadotrophin (PMSG; Folligon) and human chorionic gonadotrophin (hCG; Chorulon) were purchased from Intervet (Bendigo East, Australia). Protease inhibitor tablets were from Roche (Mannheim, Germany). Nitrocellulose was from Amersham (Buckinghamshire, UK). Unconjugated and fluorescein isothiocyanate (FITC)-conjugated anti-phosphotyrosine antibodies (clone 4G10) were from Upstate Biotechnology (Lake Placid, NY). Mowiol 4-88, monoclonal anti-hsp60 (LK-1) and goat anti-rat HRP were from Calbiochem (La Jolla, CA) and monoclonal anti-erp99 (anti-GRP94; clone 9G10) from Neomarkers (Fremont, CA). Polyclonal anti-hsp60 (H-300), polyclonal anti-erp99 (anti-GRP94, C-19) and horseradish peroxidase (HRP)-conjugated goat anti-mouse (IgG) were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgG-FITC was from Chemicon (Temecula, CA). Paraformaldehyde was supplied by ProSciTech (Thuringowa, Australia). Sulfo-NHS-LC-Biotin was obtained from Pierce (Rockford, IL). Anti-mouse IgG- and streptavidin-coated Dynabeads were from Dynal (Oslo, Norway). Ampholytes (pH 3-10) and IPG strips (7 cm, pH 3-10) were purchased from BioRad (Hercules, CA). All other reagents were from Sigma (St Louis, MO), unless otherwise stated.

Preparation of spermatozoa

Spermatozoa were obtained from adult Swiss mice (age >8 weeks; University of Newcastle Animal Facility) in HEPES-buffered Biggers, Whitten, and Whittingham media (BWW) (Biggers et al., 1971) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 μg/ml streptomycin, 20 mM HEPES buffer and 0.3% BSA. Cauda epididymides were deposited in BWW under water-saturated mineral oil at 37°C. A single tubule was cut and gentle pressure applied to release spermatozoa into the surrounding medium. Following dilution to 6×10^6 sperm/ml, cells were incubated at 37°C under an atmosphere of 5% CO_2:95% air. In some experiments, BWW was prepared without $NaHCO_3$ (BWW–HCO_3^-), without $CaCl_2$ (BWW–Ca^{2+}) or without $CaCl_2$ but with 1.7 mM $SrCl_2$ (BWW–Ca^{2+}+Sr^{2+}). BWW–HCO_3^- and BWW–Ca^{2+} were supplemented with additional NaCl to maintain an osmolarity of 300 mos^{-1}. Atomic absorption spectrometry measurements revealed that the free ionic calcium content of BWW–Ca^{2+} was 2.0±0.3 μM. BWW/PVA was prepared by substituting BSA with 1 mg/ml polyvinyl alcohol. Some samples were treated with 3 mM pentoxifylline (ptx) and 5 mM dibutyryl cyclic adenosine monophosphate (cAMP).

Preparation of oocytes

Oocytes were collected from 6-week-old Swiss mice after superovulation with 10 IU pregnant mare's gonadotrophin (PMSG) followed 48 hours later with 10 IU human chorionic gonadotrophin (hCG). Approximately 15 hours after hCG administration, oocyte-cumulus complexes were recovered and placed in 0.1% hyaluronidase for 15 minutes at 37°C. Oocytes were separated from adherent cumulus cells by gentle pipetting, washed three times in BWW and either used immediately or stored at 4°C in a high salt storage medium consisting of 1.5 mM $MgCl_2$, 0.1% dextran, 0.01 mM HEPES buffer and 0.1% polyvinyl alcohol until use. Storage of oocytes in high salt medium has been demonstrated to retain the biological characteristics of the zona pellucida (Yanagimachi et al., 1979).

SDS-PAGE and western blotting

Following incubation, spermatozoa were collected by centrifugation (500 ***g***, 3 minutes) and washed three times in BWW/PVA. Membrane-enriched protein extracts were prepared by lysing cells in 10 mM CHAPS in tris-buffered saline (TBS, pH 7.6) with protease inhibitors and 1 mM sodium orthovanadate on ice for 1 hour. Insoluble matter was removed by centrifugation (20,000 ***g***, 10 minutes, 4°C) and protein estimations performed using the DC Protein Assay Kit (BioRad) following the manufacturer's instructions.

Proteins were resolved by SDS-PAGE on polyacrylamide gels (Laemmli, 1970) followed by transfer onto nitrocellulose membranes (Towbin et al., 1979). Membranes were blocked with 3% BSA in TBS for 1 hour before being probed with monoclonal anti-phosphotyrosine antibody diluted to 1:4000 in TBS containing 1% BSA and 0.1% polyoxyethylenesorbitan monolaurate (Tween-20) for 2 hours at room temperature. Blots were washed three times in TBS containing 0.1% Tween-20 (TBS-T) followed by incubation with 1:3000 anti-mouse IgG-HRP in TBS-T containing 1% BSA for 1 hour at room temperature. Following three washes in TBS-T, proteins were detected using an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham). Western blot analysis of chaperone proteins was performed as described using monoclonal anti-hsp60 (1:1000) or monoclonal anti-erp99 (1:1000) antibodies and either anti-mouse (1:4000) or anti-rat (1:1000) IgG-HRP.

Sperm-zona pellucida binding assay

Oocytes were recovered from salt storage media, washed three times in BWW and pre-warmed to 37°C. Spermatozoa were isolated as described and incubated for 1 hour in the appropriate treatment. Pre-incubated sperm were deposited under water-saturated mineral oil at 37°C and 10-12 oocytes added to each treatment. Following a 30-minute incubation, oocytes were washed three times in BWW with a micropipette to remove loosely bound spermatozoa, mounted on slides beneath coverslips supported on 80% paraffin/20% Vaseline and compressed to around 30 μm to immobilise sperm-zona complexes. The number of spermatozoa bound to each zona was counted using phase-contrast microscopy. In some experiments, spermatozoa were preincubated for 1 hour with a 1:10 or 1:100 dilution of monoclonal anti-phosphotyrosine antibody prior to addition of zonae pellucidae. Alternatively, zonae were pre-incubated in 20 mM *O*-phospho-L-tyrosine for 1 hour prior to addition to the sperm suspension.

Immunolocalisation of proteins on fixed spermatozoa

Following incubation, spermatozoa were fixed in 1% paraformaldehyde, washed three times with phosphate-buffered saline (PBS, pH 7.4), plated onto clean glass slides coated with 0.1% poly-L-lysine and air-dried. All subsequent incubations were performed at 37°C in a humid chamber. Spermatozoa were permeabilised with 0.2% Triton X-100 for 15 minutes. Following a PBS rinse, blocking was performed using PBS containing 10% goat serum and 1% BSA for 1 hour. Cells were incubated with 1:100 anti-phosphotyrosine in PBS for 1 hour followed by three washes in PBS; goat anti-mouse IgG-FITC diluted 1:100 was applied and incubated for 1 hour. After three PBS washes, spermatozoa were mounted in 10% mowiol 4-88 with 30% glycerol in 0.2 M Tris (pH 8.5) with 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO). Cells were examined using a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Jena, Germany) and images captured using a Nikon N90S camera (Nikon, Tokyo, Japan).

Immunolocalisation of chaperone proteins was performed using the above methodology with 1:50 polyclonal anti-hsp60 or 1:50 polyclonal anti-erp99 antibody incubated overnight at 4°C followed by 1:100 goat anti-rabbit-FITC or rabbit anti-goat-FITC for 1 hour at 37°C. Blocking was carried out using a 10% solution of the appropriate whole serum.

Immunodetection of tyrosine-phosphorylated proteins on live spermatozoa

Attempts to localise tyrosine phosphorylation on live mouse spermatozoa by immunofluorescence were hampered by the fragility of spermatozoa from this species. Following several washes by centrifugation, few cells remained viable for quantification of labelling patterns. For this reason, a protocol was developed to assay live spermatozoa in a single step. Magnetic beads coated with goat anti-mouse IgG (Dynabeads M-450) were washed three times in 0.1% BSA in PBS followed by binding with monoclonal anti-phosphotyrosine for 2 hours at room temperature. Beads were washed three times with BWW and added to spermatozoa following a 1-hour incubation. The sperm/bead suspension was maintained at 37°C for a further 45 minutes with regular mixing. Wet mounts of the suspension were prepared and counterstained with 2 μg/ml propidium iodide for cell viability assessment. The percentage of viable sperm with bound anti-phosphotyrosine-coated beads was assessed by phase-contrast and fluorescence microscopy with a Zeiss Axioplan 2 microscope. Negative controls consisting of beads that were either uncoated or coated with anti-phosphotyrosine preincubated with 20 mM *O*-phospho-L-tyrosine for 2 hours at room temperature were included.

Localisation of tyrosine phosphorylation on spermatozoa bound to the zona pellucida

Zona pellucida binding was performed as described. Initial attempts to stain sperm/zona complexes directly were unsuccessful as fixation led to detachment of spermatozoa. For this reason, sperm were removed from the zona prior to staining using a fine-bore micropipette (inner diameter ~70 μm) and air-dried onto poly-L-lysine-coated slides. Cells were fixed in 1% paraformaldehyde and stained with anti-phosphotyrosine as described above.

Immunolocalisation of phosphotyrosine residues on live spermatozoa was performed directly on cells attached to the surface of the zona pellucida. Following a 45-minute incubation in capacitating media, a 1:250 dilution of FITC-conjugated anti-phosphotyrosine was added to sperm suspensions and incubated for a further 15 minutes. Droplets of spermatozoa were then deposited under mineral oil and washed zona pellucidae (prepared as described previously) added. After a 30-minute incubation, zonae were washed and mounted as before. Sperm labelling patterns were scored using fluorescence microscopy. Controls, where the antibody had been preabsorbed with 10 μg of phosphotyrosine antibody inhibitor (*O*-phospho-L-tyrosine conjugated to BSA, Sigma), were included.

Identification of sperm-surface phosphoproteins

Tyrosine-phosphorylated proteins on the surface of capacitated mouse spermatozoa were identified by purification using a biotin-streptavidin column followed by SDS-PAGE and immunoblotting. For this procedure, caudal epididymal spermatozoa were incubated for 90 minutes in either non-capacitating (BWW–HCO_3^-) or capacitating (BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP) conditions, as described previously. Cell surface proteins were labelled with 0.5 mg/ml Sulfo-NHS-LC-Biotin in BWW for 30 minutes at 37°C. Sperm were incubated for a further 10 minutes in 1 mM Tris (pH 7.6) to stop the reaction. Following three washes in BWW, cells were lysed in 10 mM CHAPS, as described. Biotinylated proteins were purified using streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin) for 1 hour at room temperature. Beads were washed three times in PBS and bound proteins eluted by boiling in 2× SDS-PAGE sample buffer (2% SDS, 2% 2-mercaptoethanol, 0.375 M Tris pH 6.8 with bromophenol blue) at 100°C for 5 minutes. Eluted proteins were applied to large-format one-dimensional 10% SDS-PAGE gels. Duplicate gels were prepared for each experiment: one stained with Colloidal Coomassie G250 (17% ammonium sulfate, 3% phosphoric acid, 0.1% Coomassie G250, 34% methanol) and the other transferred to nitrocellulose and probed with anti-phosphotyrosine as described. Surface proteins corresponding to those in which the phosphotyrosine content increased during capacitation were identified on the Coomassie-stained gel, excised, trypsin digested and sequenced by tandem mass spectrometry (MS/MS) by Proteomics International (University of Western Australia, Australia; www.proteomics.com.au). The resulting peptide sequences were used to interrogate the NCBI/BLAST Swiss-Prot database using the FASTA program (http://fasta.bioch.virginia.edu). Potential tyrosine phosphorylation sites were predicted using the NetPhos 2.0 program (http://www.cbs.dtu.dk) (Blom, 1999).

Verification of surface localisation and phosphorylation of chaperones

Proteins identified by MS/MS sequence analysis were confirmed to be present on the surface of spermatozoa by affinity purification and immunoblotting. Purified sperm surface proteins from non-capacitated (*T*=0) or capacitated (*T*=90 in BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP) populations were electrophoretically resolved and transferred to nitrocellulose as described above and the resulting immunoblots probed with anti-hsp60 and anti-erp99 antibodies.

To confirm tyrosine phosphorylation, spermatozoa were either lysed immediately (non-capacitated) or preincubated for 90 minutes in BWW (capacitated) followed by extraction in two-dimensional electrophoresis lysis buffer (4% CHAPS, 7 M urea, 2 M thiourea in 30 mM Tris, pH 8.5) on ice for 1 hour. Insoluble matter was removed by centrifugation (20,000 ***g***, 10 minutes, 4°C) and the supernatant precipitated using the PlusOne™ 2-D Cleanup kit (Amersham). The protein pellet was resuspended in rehydration buffer [4% CHAPS, 7 M urea, 2 M thiourea, 2 mg/ml 1,4-dithio-DL-threitol (DTT), 1% ampholytes (pH 3-10) and bromophenol blue]. Proteins were passively loaded onto 7-cm IPG strips (pH 3-10) by overnight rehydration at room temperature and isoelectric focusing (IEF) was performed using a Multiphor II apparatus (Amersham). IPG strips were then incubated in equilibration buffer (6 M Urea, 2% SDS, 30% glycerol, bromophenol blue in 50 mM Tris, pH 8.8) with 2% DTT at room temperature for 10 minutes then in equilibration buffer with 2.5% iodoacetamide for a further 10 minutes at room temperature. IPG strips were subsequently applied to 10% SDS-PAGE polyacrylamide gels followed by transfer to nitrocellulose as

described. Blots were probed sequentially as described above using anti-phosphotyrosine, anti-erp99 and anti-hsp60. Blots were stripped prior to reprobing in a solution containing 100 mM mercaptoethanol, 2% SDS and 62.5 mM Tris (pH 6.7) at 60°C for 1 hour, followed by several washes in TBS-T.

Statistics

Experiments were replicated with material collected from at least three different animals, and the data presented represents mean±s.e.m. Percentage data were subjected to arcsine transformation prior to performing an analysis of variance (ANOVA). Statistically significant differences between group means were tested using the Fisher's Protected Least Significant Difference (PLSD) test. Samples where $P<0.05$ were considered statistically significant.

Results

Tyrosine phosphorylation of spermatozoa bound to the zona pellucida

Anti-phosphotyrosine labelling patterns were classified according to a system proposed (Urner et al., 2001). Phosphotyrosine expression was evident either over the principle piece only (partial flagellum) or over both the midpiece and principle piece of the cell (complete flagellum) (Fig. 1A). In the free-swimming population of spermatozoa just under 15% of spermatozoa displayed partial flagellum fluorescence, and a similar proportion showed complete flagellum fluorescence (Fig. 1B). Remarkably, the percentage of spermatozoa with complete flagellum labelling among the population recovered from the zona pellucida was almost 90%. No partially labelled cells were observed in this population. This represents a highly significant ($P<0.001$) increase in the incidence of spermatozoa exhibiting complete flagellar labelling in relation to the free-swimming population of cells.

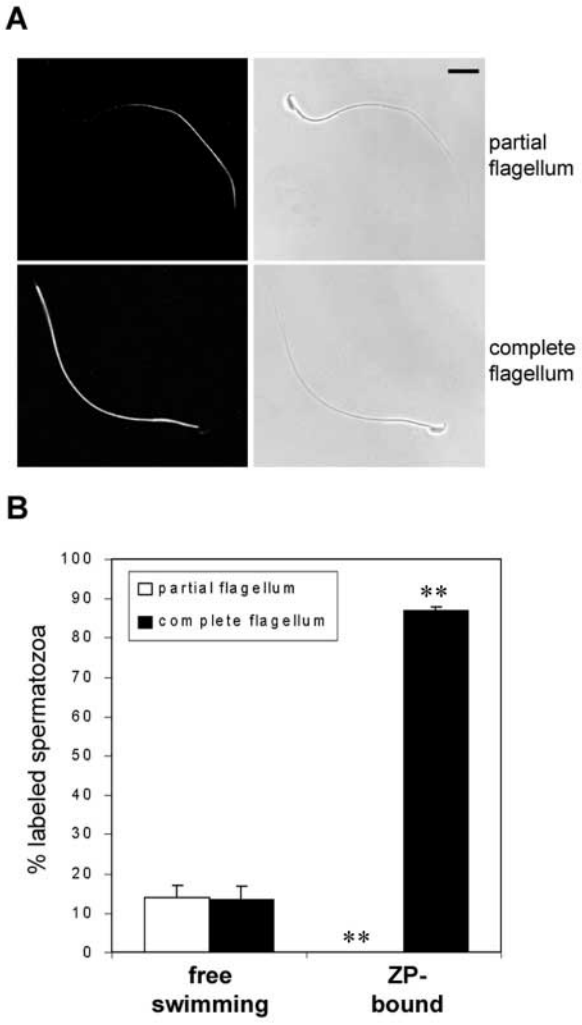

Fig. 1. Tyrosine phosphorylation of mouse spermatozoa. Cauda epididymal sperm were capacitated in BWW for 1 hour followed by 30 minutes with zona pellucidae. Sperm from the free-swimming population and recovered from the zona were fixed, permeabilised and labelled with anti-phosphotyrosine. (A) Cells displayed either partial flagellum (principal piece only) or complete flagellum labelling (midpiece and principal piece). Corresponding phase-contrast images are included. Scale bar, 10 μm. (B) Comparison of percentages of spermatozoa displaying partial (white bar) and complete (black bars) flagellum labelling in free-swimming and zona pellucida (ZP)-bound populations. The experiment was repeated four times and a minimum of 100 cells were scored for each experiment. **, $P<0.005$.

Manipulation of tyrosine phosphorylation during capacitation

The above data suggested that either the tyrosine phosphorylation status of the sperm flagellum determines the capacity of these cells to bind to the zona pellucida, that this labelling pattern is a consequence of zona binding, or alternatively that both events are mediated by a common upstream signal. In order to investigate the possible existence of a causal relationship between tyrosine phosphorylation and sperm-zona interaction, the impact of manipulating the tyrosine phosphorylation status of these cells on zona binding was investigated.

Western blot analysis of the tyrosine phosphorylation patterns exhibited by caudal mouse spermatozoa showed a 116-kDa protein to be constitutively phosphorylated. This band corresponds to the predicted size of a germ-cell-specific hexokinase previously reported in mouse spermatozoa (Kalab et al., 1994), and provides an internal loading control for the following comparisons (Fig. 2A). Incubation of sperm in complete BWW resulted in tyrosine phosphorylation of a number of proteins (45-210 kDa) as a consequence of sperm capacitation (Fig. 2A, lane 1). In keeping with previous studies (Visconti et al., 1995b), spermatozoa prepared in BWW–HCO_3^- showed low levels of phosphorylation, with the exception of the constitutively phosphorylated hexokinase (Fig. 2A, lane 2). Incubation in calcium-deficient BWW containing pentoxifylline and cAMP significantly enhanced the tyrosine phosphorylation status of the spermatozoa (Fig. 2A, lane 3). Although calcium depletion was required to fully optimise the tyrosine phosphorylation of murine spermatozoa, it has long been appreciated that extracellular calcium is required to support the physical binding of spermatozoa to the zona pellucida (Marin-Briggiler et al., 2003; Saling et al., 1978). In order to develop media that would both allow intense tyrosine phosphorylation and meet the divalent cation requirement for zona binding, calcium-deficient BWW

containing pentoxifylline and cAMP was supplemented with 1.7 mM strontium; this media supported a high level of tyrosine phosphorylation as illustrated in Fig. 2A, lane 4.

The western blot analyses presented in Fig. 2A were further supported by the immunocytochemical data showing that incubation in BWW–HCO_3^- led to a significant ($P<0.05$) reduction in the percentage of cells exhibiting phosphotyrosine expression over the entire tail compared to the BWW control (Fig. 2B). By contrast, incubation in BWW-Ca^{2+}+ptx+cAMP or BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP resulted in significant ($P<0.005$) increases in the proportion of spermatozoa exhibiting partial and complete labelling (Fig. 2B).

Tyrosine phosphorylation and zona pellucida binding

In light of these data, the above culture conditions were used to generate sperm populations expressing very different levels of tyrosine phosphorylation that could then be assessed for their ability to bind to the zona pellucida. As illustrated in Fig. 2C, cells incubated in BWW–HCO_3^- expressed low levels of flagellar tyrosine phosphorylation and exhibited a significant ($P<0.05$) decrease in zona binding compared with the controls. Conversely, the enhanced tyrosine phosphorylation status of spermatozoa incubated in BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP was associated with a significant ($P<0.005$) elevation in the zona binding capacity (Fig. 2C). There were no significant differences in the motility or vitality of spermatozoa measured following incubation in the different media (data not shown).

Tyrosine-phosphorylated proteins appear on the head of live mouse spermatozoa during capacitation

An increase in phosphotyrosine labelling on the sperm flagellum does not account for the fact that these cells have a higher affinity for the zona pellucida, an interaction mediated by proteins expressed on the plasma membrane overlying the anterior acrosome. To investigate this anomaly, the localisation of phosphotyrosine residues on live mouse spermatozoa was investigated, initially by immunofluorescence. Phosphotyrosine residues were found to appear on the head region of motile spermatozoa following capacitation (Fig. 3A). However, labelling patterns were difficult to standardise as the numerous centrifugation steps involved compromised cell viability. For this reason, an assay was developed for localisation of phosphotyrosine residues on live spermatozoa using magnetic beads coated with monoclonal anti-phosphotyrosine antibody. Using this technique, phosphotyrosine was detected on the sperm head but no other region of viable, capacitated spermatozoa (Fig. 3A).

Labeled cells showed the same general trends as the level of intracellular labelling recorded under the same treatment conditions with fixed cells, although the absolute levels of labelling were quite different (Fig. 3B). Surface tyrosine phosphorylation was observed on the head of approximately 9% of spermatozoa capacitated in complete media compared

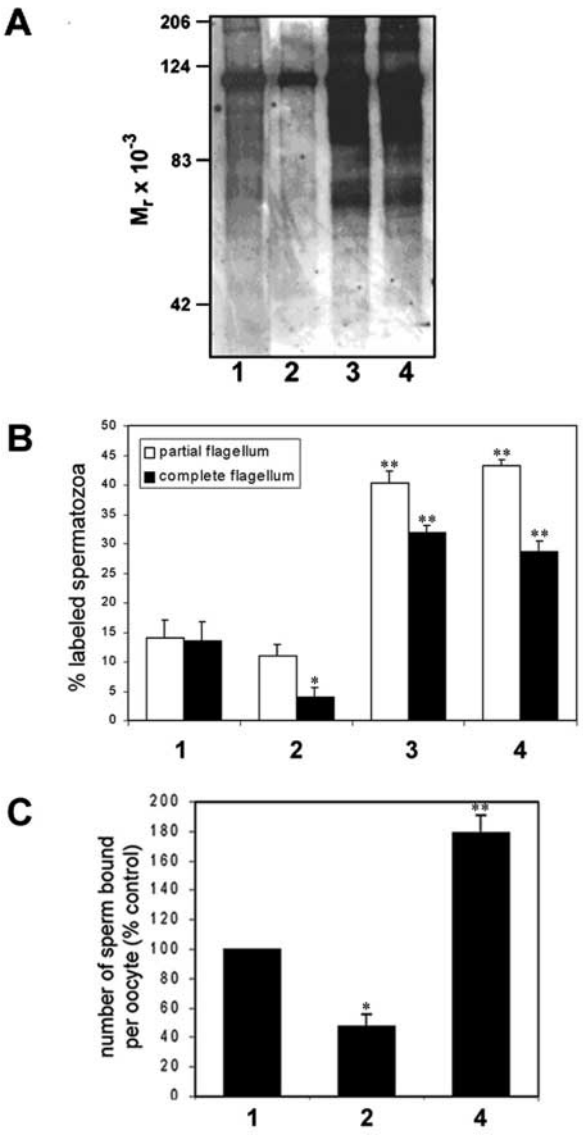

Fig. 2. Tyrosine phosphorylation and zona pellucida binding. Cauda epididymal spermatozoa were capacitated for 90 minutes in complete BWW (1), BWW–HCO_3^- (2), BWW–Ca^{2+} + pentoxifylline (ptx) + dibutyryl cyclic-AMP (cAMP) (3) or BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP (4). (A) Representative western blot of sperm proteins probed with anti-phosphotyrosine. The experiment was repeated three times. (B) Percentage of spermatozoa displaying partial (white bars) and complete (black bars) flagellum labelling following immunofluorescence with anti-phosphotyrosine. The experiment was repeated three times, with a minimum of 200 cells scored for each one. Treatment 2 led to a significant ($P<0.05$) reduction in the percentage of cells exhibiting phosphotyrosine expression over the entire tail when compared to control treated cells (1). By contrast, treatment 3 and 4 resulted in significant ($P<0.005$) increases in the proportion of spermatozoa exhibiting partial and complete labelling. *, $P<0.05$; **, $P<0.005$. (C) Correlation between tyrosine phosphorylation and zona pellucida binding. Cauda spermatozoa were prepared as described followed by a 30-minute capacitation with salt-stored oocytes. The mean number of sperm bound to each zona is expressed as a percentage of the control (treatment 1) for four repeats. Cells capacitated in solution 2 express low levels of flagellar tyrosine phosphorylation exhibited a significant ($P<0.05$) decrease in zona binding compared with the controls. Conversely, the enhanced tyrosine phosphorylation status of spermatozoa capacitated in solution 4 was associated with a significant ($P<0.005$) elevation in the zona binding capacity (Fig. 2C). *, $P<0.05$; **, $P<0.005$.

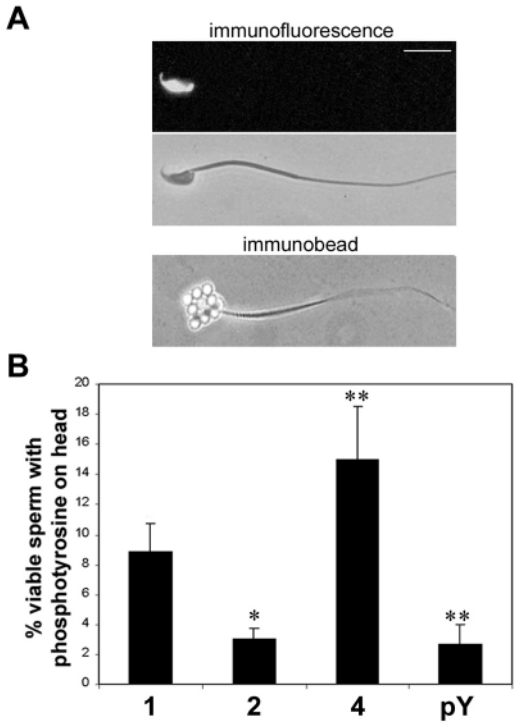

Fig. 3. Localisation of phosphotyrosine residues on live spermatozoa. Cauda epididymal spermatozoa were capacitated for 90 minutes in either complete BWW (1), BWW–HCO_3^- (2) or BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP (4). Tyrosine phosphorylation was assessed by immunofluorescence or by a 30-minute capacitation with anti-phosphotyrosine-coated magnetic beads. Controls were included where beads were preabsorbed with 20 mM *O*-phospho-L-tyrosine (pY). (A) Representative images of live spermatozoa displaying phosphotyrosine residues on the surface of the head visualised by immunofluorescence and immunobead assay. Scale bar, 10 μm. (B) Percentage of viable spermatozoa expressing phosphotyrosine residues on the head as assessed by immunobead assay. The experiment was repeated three times, with a minimum of 200 viable cells scored for each experiment. Surface tyrosine phosphorylation was observed on the head of approximately 9% of spermatozoa capacitated in complete media. This was significantly (P<0.05) decreased in uncapacitated cells (solution 2). By contrast, capacitation in solution 4 induced significantly (P<0.01) increased surface labelling compared with uncapacitated cells. Pre-incubation of phosphotyrosine beads with *O*-phospho-L-tyrosine significantly (P<0.01) reduced the labelling of cells in solution 4 to approximately 2.5%. *, P<0.05; **, P<0.01.

with 3% of cells prepared in BWW–HCO_3^-. Capacitation in BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP induced a significant (P<0.01) increase in phosphorylation of head proteins compared with the uncapacitated population (solution 2). Preincubation of phosphotyrosine beads with *O*-phospho-L-tyrosine significantly reduced the proportion of cells labelled in solution 4 (P<0.01). Labeling was also abolished following fixation in paraformaldehyde (data not shown). When compared with the data generated from studies of fixed cells (Fig. 2B) it is clear that only a subpopulation (approximately 30%) of the flagellar tyrosine-phosphorylated sperm population express phosphotyrosine residues on the external surface of the sperm head.

Live spermatozoa bound to the zona pellucida are phosphorylated on head tyrosine residues

In order to determine whether the subpopulation of cells expressing phosphotyrosine residues over the sperm head were the spermatozoa competent to bind to the zona pellucida, live sperm-zona complexes were stained with FITC-conjugated anti-phosphotyrosine. This analysis revealed that 100% of the spermatozoa bound to the surface of the zona pellucida displayed a punctate pattern of fluorescence over the head, particularly localised in the region of plasma membrane overlying the acrosome (Fig. 4A). This represents a highly significant increase (P<0.001) in the proportion of phosphorylated spermatozoa in comparison to the free-swimming sperm population (Fig. 4B). Preincubation of the antibody with anti-phosphotyrosine inhibitor completely blocked sperm labelling (data not shown).

The zona recognition region on spermatozoa is not tyrosine phosphorylated

Data indicating a relationship between tyrosine phosphorylation of sperm proteins and zona interaction prompted an analysis of whether the sperm-surface zona receptor may itself be a tyrosine phosphoprotein. The first approach used was to block phosphorylated proteins on the sperm surface with anti-phosphotyrosine (anti-pY) antibody and assess the effect on zona binding. Despite incubation of sperm with dilutions of antibody up to 1:10, no effect on zona binding was recorded when compared to untreated controls (percentage of sperm bound per zona compared with control: 1:100 anti-pY, 85±12%, n=4; 1:10 anti-pY 85±14%, n=4). To substantiate this, the complimentary experiment was performed where zonae pellucidae were preincubated with *O*-phospho-L-tyrosine to block potential sites of attachment for phosphorylated proteins. Again, no effect on sperm-zona binding was observed (percentage of sperm bound per zona compared with control: 97±7%, n=4) (data not shown).

Tyrosine-phosphorylated molecular chaperones are present on the surface of mouse spermatozoa

To identify key targets for phosphorylation in mouse spermatozoa during capacitation, the surface phosphoproteome was analysed. Tyrosine-phosphorylated proteins were isolated from affinity-purified biotinylated surface protein extracts and sequenced by tandem mass spectrometry (MS/MS). Peptide sequences obtained from three of the major phosphorylated bands, designated P1, P2 and P3 (Fig. 5A), were used to interrogate the Swiss-Prot database and matches were obtained to the following known mouse proteins: hexokinase type 1 (hk1, accession number P19376), endoplasmin (erp99, P08113) and heat shock protein 60 (hsp60, NP_034607). The alignments between the sequenced peptides and hk1 (Fig. 5B), erp99 (Fig. 5C) and hsp60 (Fig. 5D) showed 100% identity to published sequences. Analysis of the database sequence for these proteins with the NetPhos 2.0 program (Blom, 1999) revealed 3 potential tyrosine phosphorylation sites on hk1, 12 on erp99 and 5 on hsp60 (Fig. 5B-D). As hexokinase has previously been described in mouse spermatozoa (Visconti et al., 1996), characterisation of this protein was not pursued.

The localisation and phosphorylation of erp99 and hsp60 were confirmed by repeating the affinity-purification of sperm surface proteins and immunoblotting with specific monoclonal antibodies. Both erp99 and hsp60 were detected in whole lysates from both non-capacitated and capacitated spermatozoa, however, owing to the complex nature of the sperm phosphoproteome, two-dimensional electrophoresis followed by immunoblotting was used to confirm tyrosine phosphorylation. Proteins recognised by erp99 and hsp60 monoclonal antibodies aligned with spots recognised by anti-phosphotyrosine in mouse spermatozoa incubated for 90 minutes in BWW (capacitated) (Fig. 6B). Although both chaperones were detected in spermatozoa lysed at *T*=0 (non-capacitated), they were not phosphorylated in this sample (data not shown).

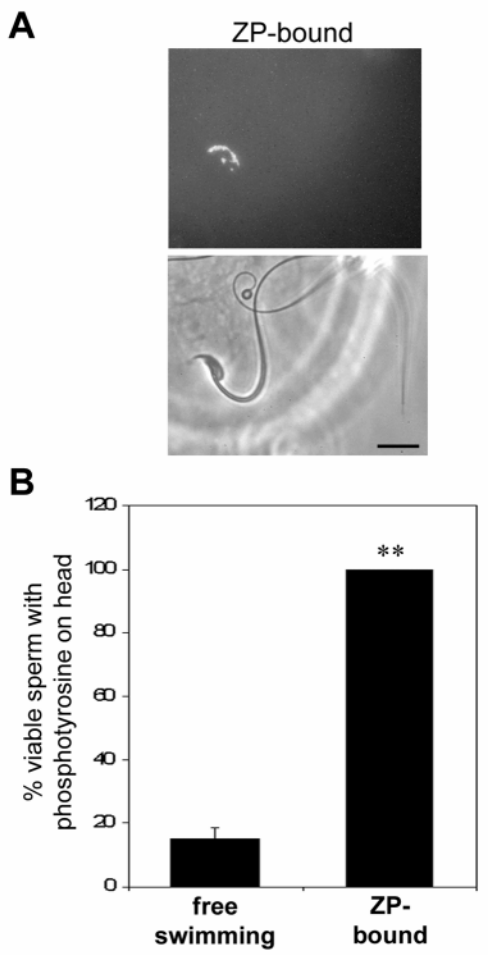

Fig. 4. Tyrosine phosphorylation of live spermatozoa bound to the zona pellucida (ZP). Cauda epididymal spermatozoa were capacitated for 1 hour in BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP followed by a 30-minute incubation with salt-stored oocytes and FITC-conjugated anti-phosphotyrosine. Sperm-ZP complexes were washed and labelling of bound spermatozoa was scored. (A) Representative fluorescent (top) and phase-contrast (bottom) images of phosphotyrosine labelling of sperm bound to ZP. Analysis revealed that 100% of the spermatozoa bound to the surface of the ZP displayed a punctate pattern of fluorescence over the head, particularly localised in the region of plasma membrane overlying the acrosome. Scale bar, 6 μm. (B) Labeled spermatozoa adhered to the ZP were compared to the free-swimming population. Phosphotyrosine was detected on the head but not the flagellum of viable spermatozoa; a highly significant increase (P<0.001) in the proportion of phosphorylated spermatozoa compared with the free-swimming sperm population was detected. The experiment was repeated three times and a minimum of 100 cells were scored for each experiment. **, P<0.001.

Hsp60 and erp99 are localised to the head of mouse spermatozoa

Hsp60 and erp99 were immunolocalised on capacitated mouse spermatozoa by indirect immunofluorescence. Both chaperones were localised to the plasma membrane region adjacent to the acrosome on the spermatozoon head (Fig. 7). There were no differences in labelling patterns observed in freshly isolated and capacitated cells, and no labelling was observed in controls in which the primary antibody was omitted (data not shown).

Discussion

The data generated in this study support a causal association between protein tyrosine phosphorylation during capacitation of mouse spermatozoa and the ability of these cells to bind to the zona pellucida. Although there have been many failed attempts to characterise the molecular basis of sperm-zona recognition, all previous approaches to this problem were predicated on the notion of a permanently expressed receptor. This study introduces the concept of a receptor complex that is assembled on the sperm surface as a consequence of the tyrosine phosphorylation events associated with sperm capacitation. This concept therefore confirms earlier biological data suggesting that sperm-egg recognition is capacitation dependent (Swenson and Dunbar, 1982). As a result of this study, we can now suggest a molecular basis for this association.

Initial anti-phosphotyrosine immunolabelling of fixed spermatozoa recovered from the zona pellucida yielded the intriguing result that almost all cells were tyrosine phosphorylated across the midpiece and principal piece. This represents a highly significant change in the phosphotyrosine status of spermatozoa compared with the free-swimming population (Fig. 1). These data suggested an important role for protein phosphorylation in gamete interaction and raised the question of whether this phosphorylation event is a cause or a consequence of zona pellucida binding. To explore this issue, capacitation media were designed to produce sperm populations in which tyrosine phosphorylation had either been suppressed or significantly enhanced, and the ability of each population to bind to the zona pellucida was assessed (Fig. 2).

In accordance with previous studies, incubation of mouse spermatozoa in complete BWW medium resulted in phosphorylation of sperm proteins from ~45 to 210 kDa (Fig. 2A, lane 1) (Ecroyd et al., 2003b; Visconti et al., 1995a). The bicarbonate ion has been demonstrated in all species studied to date to be a key component for successful capacitation (Boatman and Robbins, 1991; Gadella and Harrison, 2000; Osheroff et al., 1999; Visconti et al., 1995a). Our results confirm that omission of bicarbonate from the media leads to a global decrease in protein tyrosine phosphorylation (Fig. 2A, lane 2) and a decrease in flagellum anti-phosphotyrosine

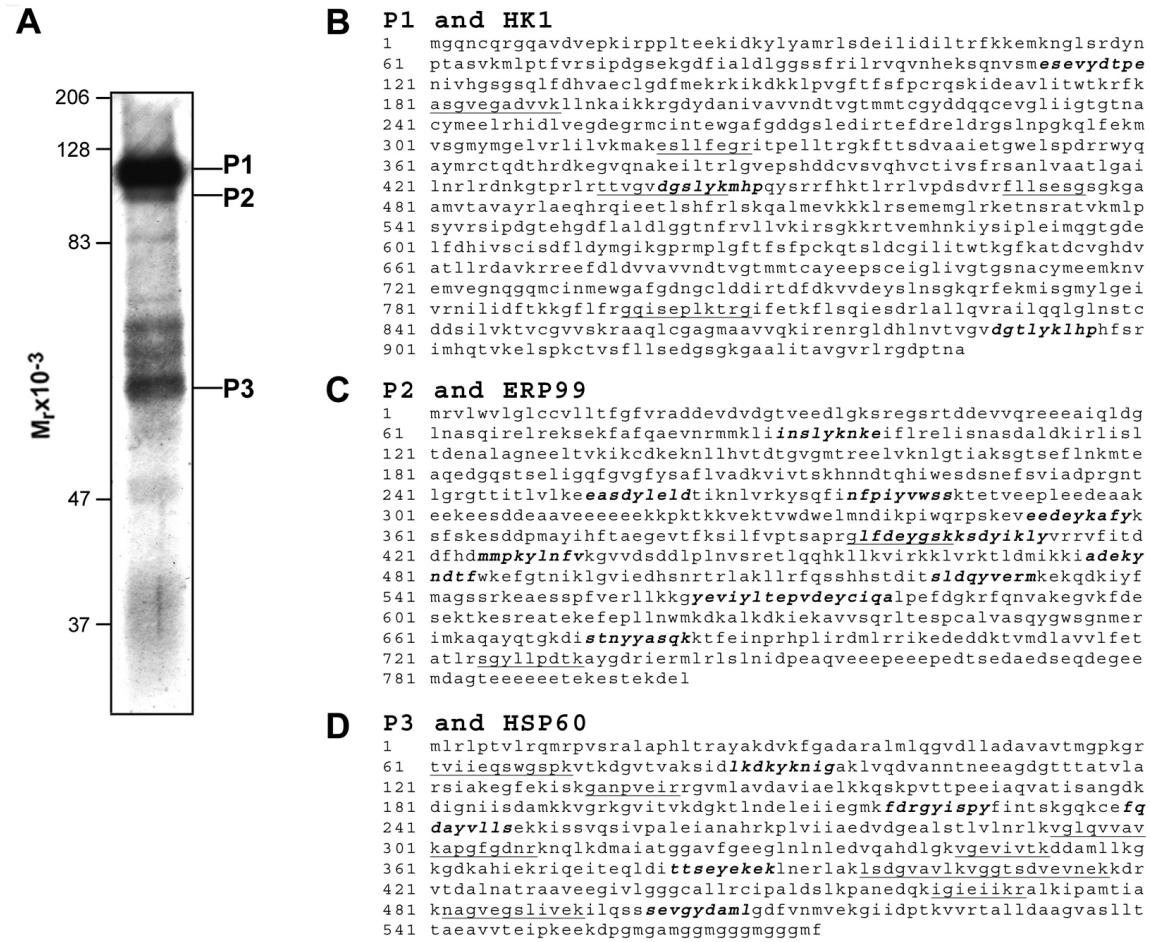

Fig. 5. Identification of tyrosine-phosphorylated proteins on the surface of spermatozoa. (A) Cauda epididymal spermatozoa were capacitated for 90 minutes in BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP. Surface proteins were affinity-purified and resolved by SDS-PAGE. Tyrosine-phosphorylated surface proteins were immunoblotted with anti-phosphotyrosine. Three major bands (P1, P2 and P3) were identified and corresponding bands sequenced from a Coomassie-stained gel by tandem mass spectrometry. (B-D) Alignment of peptide sequences from (B) P1 with mouse hexokinase type 1 (hk1, accession number P19376), (C) P2 with mouse endoplasmin (erp99, accession number P08113) and (D) P3 with mouse heat shock protein 60 (hsp60, accession number NP_034607). Sequenced peptides are underlined. Potential tyrosine phosphorylation sites predicted by NetPhos 2.0 (Blom, 1999) appear in bold italics.

fluorescence (Fig. 2B). The ability of membrane-permeable cAMP analogues and inhibitors of phosphodiesterase to stimulate significant increases in sperm protein tyrosine phosphorylation has also been well documented (Ecroyd, 2003b; Galantino-Homer et al., 1997; Nassar et al., 1999; Pommer et al., 2003; Pukazhenthi et al., 1998; Visconti et al., 1995b). In agreement with published data, we show an overall rise in tyrosine phosphorylation in response to treatment with pentoxifylline and cAMP (Fig. 2A and 2B). In order to enhance this effect further, we experimented with the use of calcium-depleted media. Data from several independent laboratories indicate that in species as diverse as the mouse, man and bull, tyrosine phosphorylation is negatively regulated by calcium (Carrera et al., 1996; Luconi et al., 1996; Vijayaraghavan et al., 1997; Ecroyd et al., 2004). Our data confirm this effect, as in medium lacking calcium and supplemented with pentoxifylline and cAMP, high levels of tyrosine phosphorylation were observed (Fig. 2A, lane 3; Fig. 2B). Nevertheless, because sperm-zona binding has an obligatory requirement for a divalent cation, calcium-deficient media are not appropriate for experiments on sperm-zona pellucida binding (Saling et al., 1978). To overcome this problem, we replaced the calcium content of BWW with equimolar strontium, as this divalent cation is known to support zona recognition (Fraser, 1987; Marin-Briggiler et al., 1999). This medium supported high levels of tyrosine phosphorylation (Fig. 2A, lane 4; Fig. 2B) and permitted an analysis on the impact of tyrosine phosphorylation on sperm-zona interaction.

Using these treatments, we demonstrated a causal relationship between the proportion of spermatozoa expressing phosphotyrosine residues and the level of sperm-zona binding. Thus, when tyrosine phosphorylation was suppressed in bicarbonate-depleted medium, sperm-zona binding was inhibited, however when phosphotyrosine expression was stimulated, zona binding was significantly enhanced (Fig. 2C). These results therefore suggest that the tyrosine phosphorylation associated with sperm capacitation is a prerequisite for sperm-zona binding. Attempts were made to

verify this finding by assessing the ability of spermatozoa to bind to the zona pellucida in response to treatment with specific tyrosine kinase inhibitors. Although tyrosine kinase inhibitors have been reported to be effective at low concentrations in somatic cells, we experienced difficulties suppressing tyrosine phosphorylation in mouse spermatozoa without compromising cell viability. The cAMP-mediated signal cascade that occurs during capacitation of mammalian spermatozoa is unique to the germ cell and thus it is feasible that inhibitors that are efficacious in somatic systems are not effective in mouse sperm.

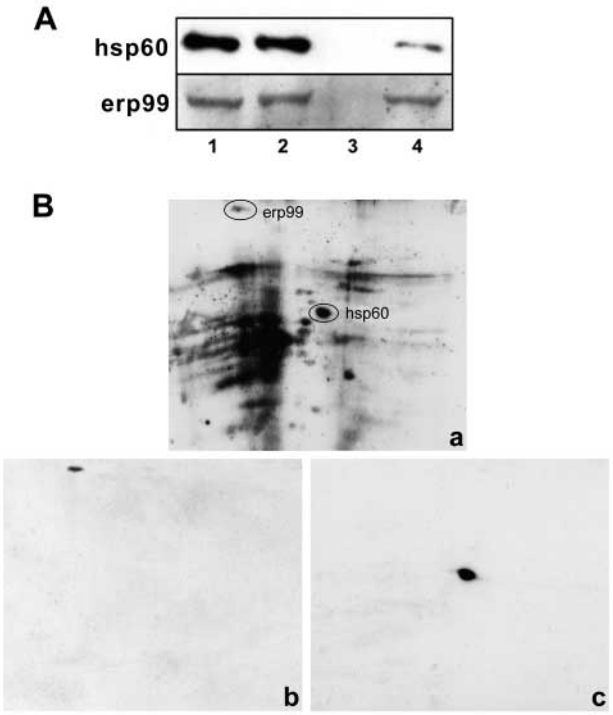

Fig. 6. Confirmation of tyrosine phosphorylation and surface localisation of erp99 and hsp60 on mouse spermatozoa. (A) Cauda epididymal spermatozoa were either freshly isolated (lanes 1 and 3) or capacitated for 90 minutes in BWW–Ca^{2+}+Sr^{2+}+ptx+cAMP (lanes 2 and 4). CHAPS detergent lysates (5 μg protein/lane) (lanes 1 and 2) and purified surface-protein extracts from 100 μg lysate (lanes 3 and 4) were prepared, resolved by SDS-PAGE and immunoblotted with anti-hsp60 and anti-erp99 monoclonal antibodies. (B) Cauda epididymal spermatozoa were capacitated for 90 minutes in BWW. Proteins were solubilised, resolved by two-dimensional electrophoresis and immunoblotted with anti-phosphotyrosine (a), anti-erp99 (b) and anti-hsp60 (c) antibodies.

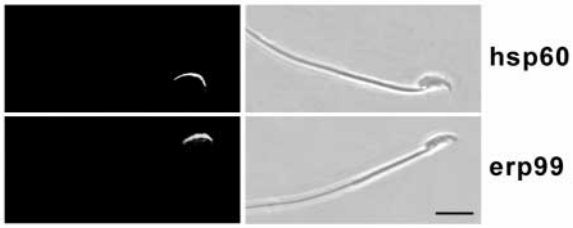

Fig. 7. Immunolocalisation of chaperones on fixed capacitated mouse spermatozoa. Representative images of mouse sperm labelled with anti-hsp60 and anti-erp99 monoclonal antibodies followed by FITC-conjugated secondary antibodies (left). Corresponding phase contrast images (right) are included. Scale bar, 10 μm.

A relationship between cAMP signalling and fertilisation is well documented (Aitken et al., 1983; Fraser, 1979; Rosado et al., 1974; Toyoda and Chang, 1974). The first evidence for a direct association between tyrosine phosphorylation and gamete interaction originated from Urner and co-workers (Urner et al., 2001). This paper described a correlation between mouse sperm flagellum phosphorylation and successful sperm-oocyte fusion. In a subsequent report, the same group reported a relationship between phosphorylation of human sperm proteins and fertilising ability (Sakkas et al., 2003). Our data support these observations by providing a link between sperm tyrosine phosphorylation and fertilising ability, isolating this effect to the initial zona pellucida binding event.

Spermatozoa are highly polarised structures, with the sperm head evolved to perform functions related to oocyte interaction and the sperm tail mediating energy production and cell motility. Localisation of phosphotyrosine residues in the sperm flagellum is therefore consistent with the onset of hyperactivated movement during capacitation (Kulanand and Shivaji, 2001; Mahony and Gwathmey, 1999; Nassar et al., 1999; Si and Okuno, 1999) however it is not consistent with a postulated role for this activity in gamete interaction. In resolving this discrepancy we have shown for the first time, phosphorylated tyrosine residues expressed on the surface of the sperm head during capacitation (Fig. 3). Furthermore, the proportion of spermatozoa displaying this pattern of expression was linked to the tyrosine phosphorylation intensity in cell lysates (Fig. 2A), the number of cells exhibiting flagellum anti-phosphotyrosine fluorescence (Fig. 2B) and with the level of zona binding (Fig. 2C). The possibility of a relationship between plasma membrane phosphoprotein expression and zona binding has been alluded to in other mammalian species (Flesch et al., 1999; Flesch et al., 2001; Tardif et al., 2001). However, this is the first report of the appearance of tyrosine-phosphorylated proteins on the surface of live mouse spermatozoa during capacitation. These observations raise important questions about the functional competence of this subgroup of capacitated cells, which represent about one third of the total capacitated population.

The importance of this novel phosphorylation pattern in zona binding was emphasised in imaging experiments where every spermatozoon bound to the zona pellucida was found to express phosphorylated tyrosine residues on the head (Fig. 4). This represents a significant increase compared to the occurrence of this labelling pattern in the free-swimming population. These results are consistent with the notion that this phosphorylation pattern is a prerequisite event that facilitates zona recognition and ultimately renders the spermatozoon capable of fertilisation.

These data therefore suggest that superficially expressed tyrosine phosphoproteins may be involved in sperm-zona recognition. A previous report describes a reduction in zona pellucida binding when live human spermatozoa are pre-incubated with monoclonal anti-phosphotyrosine antibody, suggesting that tyrosine phosphoproteins may be directly involved in gamete adhesion (Kadam et al., 1995). We have performed the analogous experiment in the mouse, with contrary results. Following pre-incubation of mouse spermatozoa with serial dilutions of anti-phosphotyrosine

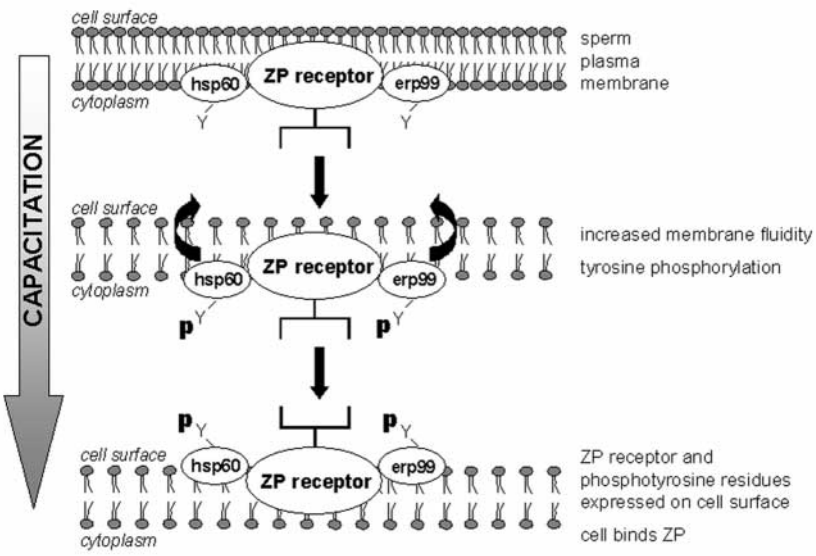

Fig. 8. Proposed model for the involvement of tyrosine-phosphorylated molecular chaperones in the acquisition of mammalian sperm fertilizing ability. We hypothesise that a zona pellucida (ZP) receptor complex present in the plasma membrane of spermatozoa might encompass a ZP-binding molecule and also the chaperone proteins heat shock protein 60 (hsp60) and endoplasmin (erp99). In freshly ejaculated spermatozoa, these molecules might be on the cytoplasmic side of the membrane. During capacitation, cholesterol efflux from the membrane promotes increased fluidity, which facilitates changes in protein distribution. Tyrosine phosphorylation of hsp60 and erp99 on the inner surface of the membrane might activate this receptor complex and allow conformational changes such that the chaperone proteins and ZP binding molecule are exposed to the cell surface. This would account for the appearance of phosphotyrosine residues observed on the sperm head following capacitation, and the association of this event with the attainment to recognise and bind the ZP.

antibody, no effect on zona pellucida binding was recorded. In addition, pre-incubation of zonae with an excess of phosphotyrosine did not influence sperm binding. This suggests that although tyrosine-phosphorylated proteins appear to be involved in gamete interaction, the specific region responsible for zona recognition and adhesion in mouse spermatozoa does not contain phosphotyrosine.

If tyrosine-phosphorylated proteins are not directly involved in sperm-zona recognition, they may be instrumental in the assembly of receptor complex on the surface of the spermatozoa that mediates zona recognition. The male gamete is a transcriptionally inactive cell, relying on post-translational modifications to activate proteins in response to exogenous signals, such as those originating from the oocyte. Phosphorylation of a protein within the zona pellucida-receptor complex during capacitation may trigger conformational changes that expose or activate zona pellucida binding domains and result in the formation of a functional surface receptor. Such conformational changes may include removal of surface-associated masking molecules or re-orientation of key recognition peptides to the surface of the cell.

Until recently, the only target for tyrosine phosphorylation identified in mouse spermatozoa was a constitutively phosphorylated germ-cell specific form of hexokinase on the surface of the cell (Kalab et al., 1994; Visconti et al., 1996). The first report of a protein increasing in phosphotyrosine content during capacitation came from Ecroyd and colleagues, who identified heat shock protein 90 as a target for phosphorylation in mouse spermatozoa (Ecroyd et al., 2003a). The realisation that proteins on the surface of mouse spermatozoa become phosphorylated during capacitation prompted an analysis of the surface phosphoproteome of these cells. Three major proteins were identified: hexokinase, heat shock protein 60 and endoplasmin (Fig. 5). The identification of hexokinase acted as a valuable internal control. In addition, we have reported for the first time the expression of two molecular chaperones on the surface of mouse spermatozoa, heat shock protein 60 (hsp60) and endoplasmin (erp99). Sequence analysis revealed a number of potential tyrosine phosphorylation sites on each protein (Fig. 5).

The presence, phosphorylation and localisation of these molecular chaperones in mouse spermatozoa were confirmed by western blot analysis and immunocytochemistry. Both erp99 and hsp60 were detected in detergent lysates and affinity-purified surface proteins preparations from capacitated mouse spermatozoa following immunoblotting (Fig. 6A) and were aligned with tyrosine-phosphorylated spots on 2D gels (Fig. 6B). Furthermore, both were found to be localised to the plasma membrane region of the head overlying the acrosome in both freshly isolated and capacitated spermatozoa (Fig. 7). However, only following the phosphorylation events associated with sperm capacitation were these proteins found to form a superficially-located pool of protein that can be biotinylated in intact cells (Fig. 6A).

Although chaperones were initially thought to be restricted to organelles within the cell, reports are emerging describing secondary roles on the cell surface. Calnexin, calreticulin, gp96, GRP74, GRP78, HSP72, HSP70, HSP60, HSP54, HSP27 and protein disulfide isomerase have now been reported on the surface of a range of cell types including cancer cells, fibroblasts and blood cells (Akagi et al., 1988; Altmeyer et al., 1996; Essex et al., 1995; Ferrarini et al., 1992; Goicoechea et al., 2000; Okazaki et al., 2000; White et al., 1995; Wiest et al., 1995; Wiest et al., 1997). A recent study has revealed the major proteins on the surface of certain cancer cells to be molecular chaperones (Shin et al., 2003). Furthermore, the first report of chaperones on the gamete surface was recently published (Calvert et al., 2003). GRP94, GRP78, calreticulin and HSP90 were identified on the surface of the mature mouse oocyte, providing the first suggestion of a role for chaperones in mammalian fertilisation.

Both erp99 and hsp60 have been reported in the testis (Aguilar-Mahecha et al., 2001; Meinhardt et al., 1995; Werner et al., 1997), however, this is the first report of these chaperones in spermatozoa. Heat shock protein 90 was recently identified on both human and mouse sperm, and identified as a key phosphoprotein activated during capacitation (Ecroyd et al., 2003a; Ficarro et al., 2003). The current data provide novel evidence for tyrosine-phosphorylated chaperone proteins on the surface of mouse spermatozoa, and the finding of

endoplasmin and heat shock protein 60 in the mature gamete suggests a possible post-spermatogenic role for these proteins.

We postulate that endoplasmin and heat shock protein 60 may be involved in the receptor-mediated interactions that control fertilisation. The localisation of these proteins on the plasma membrane overlying the acrosome combined with their tyrosine phosphorylation during capacitation raises questions regarding their potential involvement in the recognition and binding events involving the capacitated spermatozoon and the zona pellucida. One intriguing possibility is that chaperone proteins may be involved in the reorientation of sperm plasma membrane proteins during capacitation such that the putative zona pellucida receptor complex is available for interaction with its ligand. One potential model is presented in Fig. 8. According to this model, cholesterol efflux from the sperm plasma membrane during capacitation promotes increased fluidity and enhanced protein redistribution (Cross, 1998). It is possible that prior to capacitation the zona pellucida receptor complex is not exposed on the surface of the spermatozoon. Following ejaculation and initiation of cAMP signalling, changes in plasma membrane lipid architecture in concert with activation of molecular chaperones by tyrosine phosphorylation may facilitate conformational changes that result in the zona recognition complex being exposed on the cell surface. It is important to note that the phosphorylation of a tyrosine residue essentially changes a hydrophobic residue to a hydrophilic one. Such a change may constitute a possible mechanism for reorientation of the complex in the membrane leading to surface exposure of the receptor. Alternatively, capacitation may be associated with the removal of surface-associated proteins ('decapacitation factors') such that the receptor molecule is exposed. This reconfiguration would not only prepare the spermatozoon for gamete recognition but also result in the surface exposure of phosphotyrosine residues, a phenomenon reported for the first time in this paper. The model presented in Fig. 8 is a simplistic one and it is likely that a number of cofactors would be involved in the formation of a chaperone-client protein complex, such as is the case in somatic models.

This study elucidates some of the key biochemical features of sperm-egg interaction in the mouse and suggests a role for molecular chaperones in this process. Despite phosphotyrosine expression being recognised as an important correlate of capacitation, the virtual absence of unphosphorylated sperm among the population bound to the zona pellucida has not previously been appreciated. We also demonstrate the localisation of phosphotyrosine residues on the exterior of the capacitated spermatozoon. These data establish that phosphotyrosine expression per se is not directly responsible for sperm-egg recognition but is indirectly involved, possibly by directing the assembly of a zona-receptor complex on the sperm surface. Identification of phosphorylated endoplasmin and heat shock protein 60 on the plasma membrane of the mouse sperm head following capacitation is consistent with a role for these chaperones in the assemblage of just such a complex. This is a novel concept that will inform future studies on the mechanisms by which capacitation creates a functional spermatozoon, with implications for both the diagnosis of male factor infertility and male-oriented methods of fertility control.

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